RESEARCH PAPER

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Development of a Vero cell DNA reference standard for residual DNA measurement in China

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Abbreviations: Ch.P, Pharmacopoeia of the People's Republic of China; WHO, World Health Organization; BSA, Bovine serum albumin

This collaborative study developed a Vero cell DNA reference for standardizing dot blot hybridization, an assay widely employed to measure residual DNA contents of viral vaccines prepared with Vero cells. High purity of Vero cell DNA was extracted and characterized by Hind III enzyme digestion and DNA sequencing. Then, with a cooperative calibration, the concentration of Vero cell DNA reference bulk solution was determined (64.0 ± 1.9 μg/mL, OD_{260}/OD_{280} = 1.87) and diluted (40 ng/mL) with Tris-EDTA buffer containing bovine serum albumin as freeze-dried excipients. With industrial filling apparatus, the diluted bulk was loaded into ampoules (0.5 mL each) which were heat sealed after nitrogen filling. Finally, a collaborative study showed that the Vero cell DNA reference could reach a sensitivity of 1 to 5 pg/dot and maintained good stability after accelerated destruction test. The successful establishment of the Vero cell DNA quantitative reference will facilitate the standardization of dot blot hybridization for testing residual host cell DNA.

Introduction

The Vero cell line is a continuous cell lineage derived from kidney epithelial cells of an African green monkey.[1] Being easy to cultivate and suitable for mass production, Vero cells were initially used for vaccine preparation in the 1980s and has been recommended by WHO to be one of the cell substrates for producing vaccines for human use. Vero cells are being widely employed in the preparation of viral vaccines particularly in developing countries, and account for approximately 80% of the global production of viral vaccines.[2,3] In China, for example, the rabies vaccine for human use prepared by Vero cells takes more than 90% of the market share with the annual usage exceeding 10 million doses. Given Vero cells belong to the continuous cell lineage and were shown to be positive for carcinogenicity test after over 170 passages, their genomic DNA carried in vaccines could be oncogenic and hence poses a potential risk for human carcinogenesis. A quality control is therefore needed to limit the residual DNA of Vero cells contained in viral vaccines. Currently, the standard limits adopted by various countries (U.S. Pharmacopoeia, European Pharmacopoeia and Pharmacopoeia of the People's Republic of China) for viral vaccines are mainly three grades: 10 ng/dose, 100 pg/dose and 10 pg/dose.

Of the techniques available for testing residual DNA of Vero cells, molecular hybridization is the one most commonly used in China and other developing countries. The assay is based on the binding of denatured DNA probes to their complementary base sequences fixed on a supporting membrane. The DNA probes are specific fragments of Vero cell DNA labeled with digoxigenin, biotin, or radioisotope. After hybridization and washing, the signal is visualized by luminescence, autoradiography or other methods. Semi-quantitatively, the amount of residual DNA in the unknown sample can be obtained by comparing signal intensity with that of known DNA standards. The assay is straightforward and the detection sensitivity can reach a region 1 to 10 pg. So far, this technique has been extensively employed for economical and practical reasons, but it was also known to have high degree of inter-laboratory variation. The variability can be attributed to numerous factors that are introduced in the multiple steps of the experimental procedure.[4] An important but often ignored factor, however, is the absence of a Vero cell DNA reference standard. Generally, the laboratories or vaccine manufacturers prepare their own batch of Vero cell DNA and use as reference after UV absorption reading. As a result, this practice gives rise to systemic errors and consequently makes the test results less comparable between laboratories. To standardized hybridization assays, we have established a reference standard of Vero cell DNA suitable for dot blot analysis and our results indicate that it satisfies the sensitivity and precision required for hybridization assay. A detailed report is presented below.

Results

Preparation of the raw material for Vero cell DNA reference. The genomic DNA of Vero cells was extracted and first analyzed

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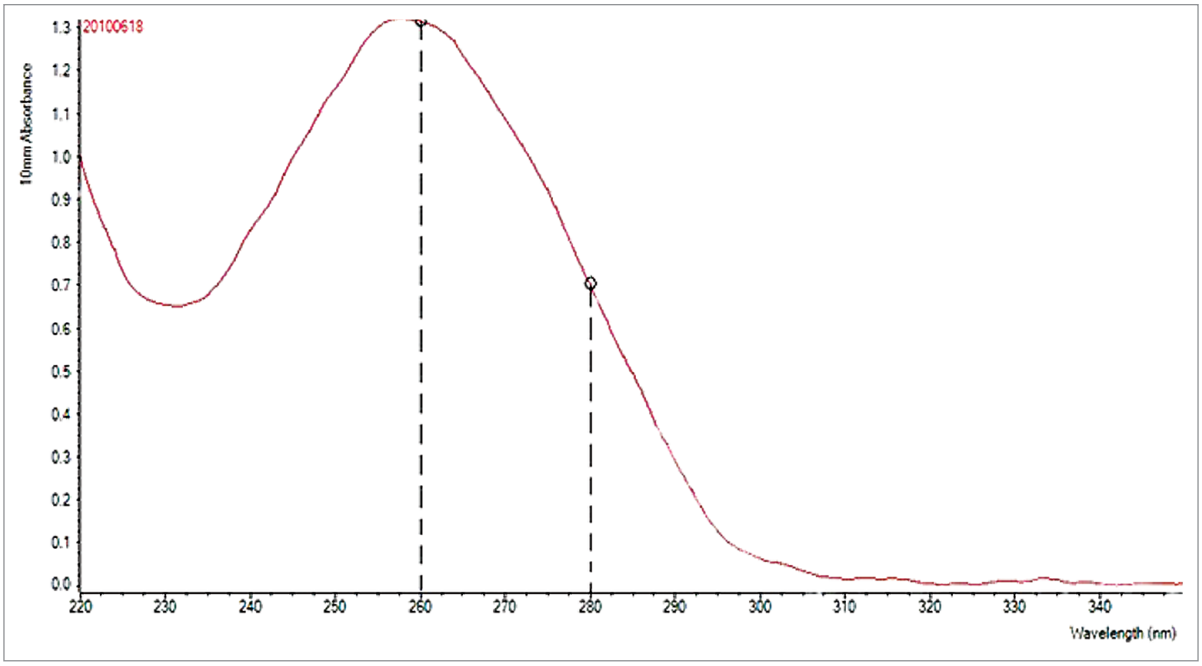

Figure 1. Analysis of Vero cell genomic DNA with full wavelength scanning (220nm—340nm).

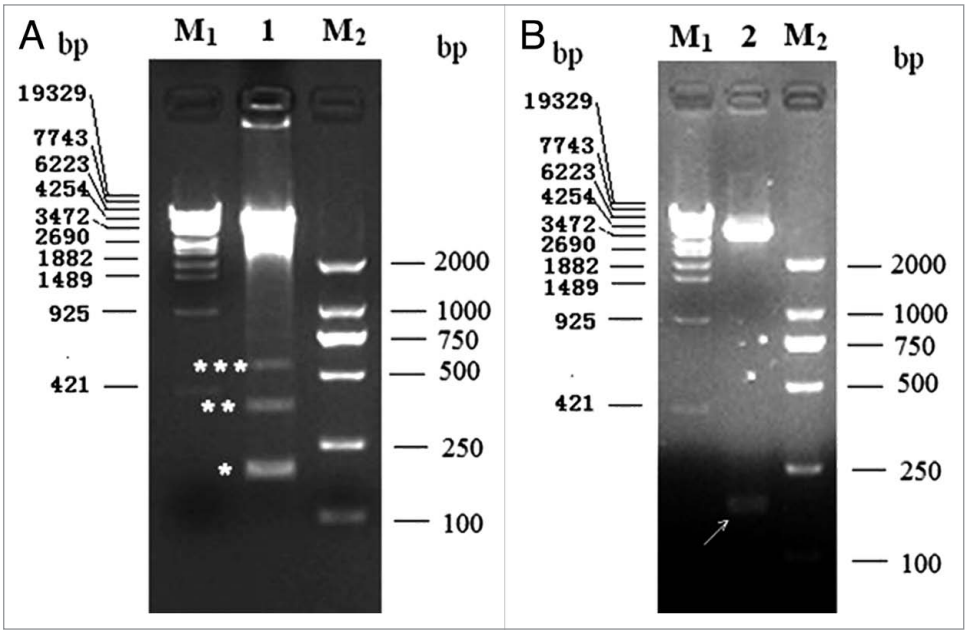

Figure 2. Identification of Vero Cell DNA. (A) Analysis of Vero cell genomic DNA with Hind III digestion. (B) Enzymatic cleavage of pET30a-172 recombinant plasmid with Hind III. (M1) 1 kb DNA ladder 1; M2:100 bp DNA ladder. Lane 1, Vero cell DNA after HindIII digestion. * 172 bp, **: 344 bp, *** 516 bp. Lane 2, pET30a-172 recombinant plasmid after HindIII digestion. Arrow points to the fragment corresponding to 172 bp.

by an UV-visible spectrophotometer at wavelengths from 200 to 800 nm. **Figure 1** shows that the extracted DNA materials were of high quality and purity, evidenced by a strong absorption peak at 260 nm as well as a OD_{260}/OD_{280} ratio of 1.8 to 2.0.

In order to enlarge the observation of 260 nm absorption peak shape **Figure 1** shows a cut diagram with wavelengths from 220 nm to 345 nm.

The genomic DNA of Vero Cells was also analyzed by 1.2% agarose gel electrophoresis after digestion with Hind III restriction endonuclease. According to reference 5, Vero cell genomic DNA produces repeated DNA fragments of 172 bp, 344 bp and 516 bp after Hind III digestion. **Figure 2A** shows that the digestion produced a ladder of DNA fragments with three bands that migrated at the front displaying sizes close to 172 bp, 344 bp and 516 bp. This pattern of cleavage was consistent with previous report.[5]

Finally, the origin of DNA was validated by DNA sequencing. The DNA from the band corresponding to 172 bp was cloned into pET30a (+) vector to generate pET30a-172 (**Fig. 2B**). Sequencing the plasmid indicated the insert showed an identity of 99.8% to that of Vero cell genomic DNA (GenBank: V00145.1). Two mismatches observed were found to be heterozygous mutation. Taken together, all these results indicate that the genomic DNA prepared were of high quality and purity, of Vero cell DNA origin,

Table 1. The calibration results of concentration and purity of DNA reference bulk

Lab No.	$OD_{260\ nm}$	$OD_{280\ nm}$	OD_{260}/OD_{280}	DNA concentration (μg/mL)
1	1.245	0.674	1.85	/
2	1.265	0.671	1.90	/
3	1.314	0.707	1.86	/
4	1.231	0.646	1.91	/
5	1.333	0.713	1.87	/
6	1.234	0.662	1.86	/
7	1.310	0.693	1.89	/
8	1.294	0.709	1.82	/
9	1.256	0.668	1.88	/
10	1.324	0.715	1.85	/
Mean	1.281 ± 0.039*	0.686 ± 0.025*	1.87 ± 0.03*	64.0 ± 1.9*
95% C.I.**	1.253~1.308	0.668~0.703	1.85~1.89	63~65

Notes: *, mean ± 1SD; **, confidence interval. One-sample T test ($p < 0.001$).

and satisfies the requirements of Ch.P and WHO for DNA standard samples.[6]

Concentration calibration of Vero cell DNA reference bulk solution. Ten certified, independent laboratories were invited to collaboratively calibrate the concentration and purity of the DNA bulk. Data were collected from all participating laboratories and were analyzed by SPSS11.5 software. The arithmetic mean, 64.0 ± 1.9 μg/mL, was considered the final concentration of the bulk of Vero cell DNA standard. The average ratio of OD_{260}/ OD_{280} was determined to be 1.87, well within the range of 1.8 to 2.0, indicating high purity of the DNA preparation (**Table 1**). In the following experiments, 64.0 μg/mL was used for calculation and dilution purpose.

Ampoule filling of the Vero cell DNA reference. The prepared DNA was diluted to 40 ng/mL and dispensed into the 2 mL ampoules (0.5 mL each). After freeze-drying, the Vero cell DNA reference appeared to be milky-white loose body (**Fig. 3**). The sample appeared to be clear and transparent liquid after reconstitution with water. The time required for full reconstitution was less than 1 min. The residual moisture was determined to be 2.5%, and the pH was 8.3. The filling precision was ranged from -0.1% to 0.4% at the beginning stage, -0.3% to 0.5% in the middle stage, and at 0.6% in the end stage of dispensation. Overall, the precision was within ± 1% (**Table 2**), which complied with the requirements for standards in Ch.P. In total, 4,000 ampoules of Vero cell DNA reference were obtained.

Applicability validation of Vero cell DNA reference. Ten laboratories participated in the collaborative study to validate the applicability of Vero cell DNA reference. Among ten laboratories, five detected signals from 1 pg/dot, seven detected signals from 5 pg/dot, and ten detected signals from 10 pg/dot. Therefore, the sensitivity of the standards could reach 1 pg/dot (**Table 3**, **Fig. 4**). Additionally, there was good linearity observed between the signals generated and the quantity of DNA loaded per dot (**Fig. 4**).

For accelerated destruction test, there was no difference observed between DNA samples treated at 37°C (2 weeks, or 4 weeks) and those stored at -20°C (normal storage). The sensitivity of assay remained 1 pg/dot without significant changes, indicating high stability for this batch of reference standards (**Fig. 5**).

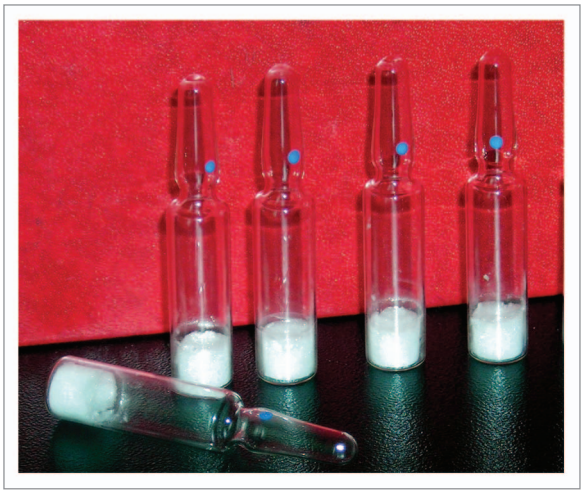

Figure 3. Appearance of the freeze-dried product of Vero cell DNA reference.

Discussion

The huge market share of viral vaccines prepared with Vero cells prompts the drug regulatory agencies of China to assess the residual DNA contents of Vero cells in vaccines for human use considering the potential risk for carcinogenesis. To control test variability between laboratories, this study was initiated to develop and evaluate a Vero cell DNA reference for standardizing hybridization assays, specifically for dot blot assay which is widely employed in China and other developing countries. For preparing this reference, WHO guidelines for standard materials were referred to and strictly followed as few reports are available about preparing DNA reference standards, especially those for quantitative analysis.[6-9]

Table 2. Filling precision of DNA reference

Loading Needle	Beginning Stage		Middle Stage		End Stage	
	Actual load (g)	Dispensing precision*(%)	Actual load (g)	Dispensing precision*(%)	Actual load (g)	Dispensing precision*(%)
1	0.5100	-0.06	0.5098	-0.10	0.5145	0.82
2	0.5102	-0.02	0.5122	0.37	0.5133	0.59
3	0.5123	0.39	0.5066	-0.73	0.5136	0.65
4	0.5093	-0.20	0.5116	0.25	0.5129	0.51
5	0.5099	-0.08	0.5139	0.71	0.5141	0.74
6	0.5131	0.55	0.5145	0.82	0.5140	0.73
7	0.5124	0.41	0.5144	0.80	0.5137	0.67
8	0.5100	-0.06	0.5132	0.57	0.5125	0.43
9	0.5126	0.45	0.5120	0.33	0.5144	0.80
10	0.5144	0.80	0.5122	0.37	0.5120	0.33
11	0.5111	0.16	0.5099	-0.08	0.5118	0.29
12	0.5120	0.33	0.5111	0.16	0.5122	0.37
Mean	/	-0.1~0.4	/	-0.3~0.5	/	0.6

Note: *, the theoretical quantity is 0.5103 g obtained after multiplying 0.5 mL with 1.0206 g/mL, the density of the DNA reference.

Table 3. Summary of the sensitivity result of the Vero cell DNA reference standards

Lab No.	10 ng/dot	1 ng/dot	500 pg/dot	100 pg/dot	50 pg/dot	10 pg/dot	5 pg/dot	1 pg/dot	0.1 pg/dot
1	+	+	+	+	+	+	+	/	/
2	+	+	+	+	+	+	+	+	/
3	+	+	+	+	+	+	/	/	/
4	+	+	+	+	+	+	+	+	/
5	+	+	+	+	+	+	+	+	/
6	+	+	+	+	+	+	+	+	/
7	+	+	+	+	+	+	+	/	/
8	+	+	+	+	+	+	/	/	/
9	+	+	+	+	+	+	+	+	/
10	+	+	+	+	+	+	/	/	/
Total (%)	100	100	100	100	100	100	70	50	0

Note: "+," represents signal observed; "/," represents no signal observed.

To meet the requirements for DNA standard, following steps have been taken to ensure the quality of Vero cell DNA as well as the accuracy of the final products. First, the bulk DNA solution was of high purity, indicated by a single peak at 260 nm as well as an OD_{260}/OD_{280} ratio of 1.8 to 2.0. The origin of the DNA was also confirmed to be of Vero cells after enzymatic digestion and DNA sequencing. Second, the concentration of the bulk solution (64.0 μg/mL) was collaboratively calibrated by ten independent, certified laboratories (**Table 1**). Third, the diluted DNA was carefully filled into ampoules under rigorous precision control (**Table 2**). The precision was within ± 1%, which complies with the requirements for standards in Ch.P 2010 and that for WHO.[6] Finally, the maintenance of Vero cells as master and working cell banks ensures the consistency of DNA references made in batches in terms of passage numbers even for later preparation. The collaborative study demonstrated the DNA reference was successfully prepared. With a high sensitivity (1~5 pg/dot) and stability (**Table 3**, **Figs. 4 and 5**), it has satisfied the requirements for testing residual DNA of Vero cells by dot blot hybridization.

The presence of nuclease-free BSA in the reference has many advantages. It can minimize the loss of DNA during filling process through blocking non-specific binding of DNA to filling line. DNA readily adheres to objects which can lead to significant loss of DNA during industrial filling, particularly at low concentration. On the other hand, it can also decrease loss of DNA after reconstitution by reducing non-specific binding of DNA to wall of ampoules. Importantly, it can enhance test reliability by reducing errors resulting from DNA recovery rate during DNA extraction step. This is achieved by processing DNA standard in parallel with vaccine samples. As a result, DNA extraction can be performed with inexpensive phenol-chloroform method, which will promote the dot blot assay in quality control of vaccines in domestic industries and other developing countries.

In conclusion, the reference DNA of Vero cells we established has fully complied with the Chinese Pharmacopeia specification for the precision of filling, sensitivity and stability for the determination of residual DNA of Vero cells in vaccine products by dot blot assay. After it was approved by the National Standard Agency of China (code 2011–0045), the reference has been applied in National Control Laboratories and domestic industries in China for more than one year. It has been playing an important role in the detection of residual DNA from viral vaccines produced with Vero cells substrate. Further studies are ongoing to investigate the quality of the standard throughout the life-time of the product as well as to gather stability data. Since no similar reference product has been reported from WHO and other countries/regions, this study can serve a guideline for preparing DNA reference standards in quality control of viral vaccine.

Materials and Methods

Raw material preparation for Vero cell DNA reference. *Cell culture.* Vero cells (ATCC, Passage 128) were cultured in MEM medium (Gibco) and a Master Cell Bank (lot: 20020102) was established after six consecutive passages. One aliquot of cells from this bank was further cultured three more passages to set up Working Cell Bank (lot: 20090101). Both banks of cells were stored in liquid nitrogen.

DNA extraction. One vial of Vero cells from the Working Cell Bank was revived and passaged five times. Then, confluent monolayer cells were trypsinized and harvested by centrifugation at 8000 RPM for 10 min. The cells were washed twice and resuspended in 50 mL PBS. The cell suspension was used for isolating genomic DNA according to the instructions provided by Blood and Cell Culture DNA Maxi Kit (Qiagen), and the DNA was dissolved in 100 mL Tris-EDTA (TE) buffer (pH8.0).

DNA identification. The genomic DNA prepared above was first analyzed with a spectrophotometer for full wavelength scanning (200 nm to 800 nm). Next, 50 μl of DNA was digested with Hind III restriction enzyme (NEB), electrophoresised on a 1.2% agarose gel, and stained with ethidium bromide. Then the band corresponding to 172 bp was cut,[5] and the DNA fragments were purified and ligated with pET-30a (+) vector (Invitrogen). The ligation product was used to transform DH5α cells (Invitrogen) and positive clones were selected on LB plate containing 50 μg/mL kanamycin (Sigma-Aldrich). The plasmid obtained was sequenced and a BLAST search was performed against Vero cell DNA sequences stored in the GenBank database.

Concentration calibration of Vero cell DNA reference bulk solution. Ten independent, certified laboratories were selected to collaboratively calibrate the concentration of Vero cell DNA reference bulk solution using UV absorption method. The optical path was set at 10 mm for the quartz cuvette, and the absorbency values at 260 nm and 280 nm wavelengths were recorded. The DNA concentration (μg/mL) was calculated by multiplying 50 with the absorbency value at 260 nm. The final concentration of Vero cell DNA reference bulk solution was determined to be the arithmetic mean of values reported from participating laboratories after statistical processing (SPSS, Version 11.5).

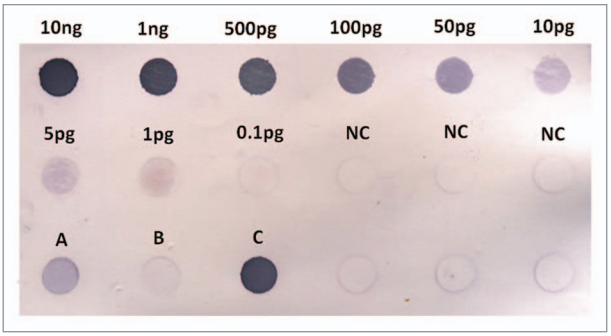

Figure 4. Sensitivity anaysis of Vero cell DNA reference by dot blot assay. NC, Negative Control; (A, B and C) Blind samples.

Ampoule filling of the Vero cell DNA reference. The calibrated bulk solution of Vero cell DNA was diluted with sterilized 1.5% BSA (Merck, dissolved in TE buffer) to reach a final concentration of 40 ng/mL. The solution was dispensed into 2 mL ampoules (0.5 mL each) by an industrial filling line (Wuhan Institute of Biological Products, GMP certified). Before the actual filling, 1.5% BSA solution was used to block non-specific binding sites on the filling line. Subsequently, the bulk solution of Vero cell DNA was diluted and dispensed into ampoules, with the first 1,000 ampoules being used to adjust the filling precision, followed by actual filling. The filling precision was calculated according to the formula: Precision = 100% × (Actual quantity - Theoretical quantity)/Theoretical quantity. The Actual quantity represents the weight of a filled ampoule after deducting the weight of the empty ampoule, while the theoretical quantity was the calculated weight in a volume of 0.5 mL after multiplying the density of the

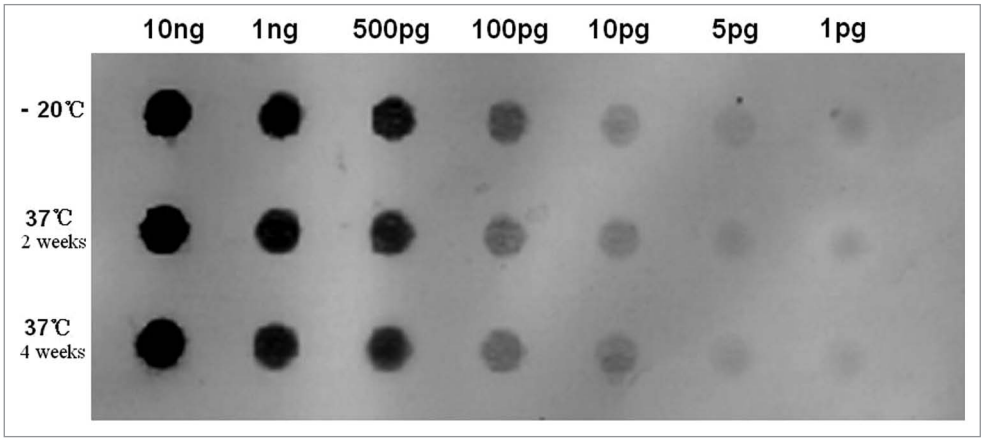

Figure 5. Stability analysis of Vero cell DNA reference by dot blot assay.

Appendix. List of participating laboratories

Laboratories	Operator	Address
Wuhan Institute of Biological Products	Prof. Bangling Yang and Dr. Yanhong Shi	9 Linjiang Avenue, Wuchang, Wuhan city, PR China 430060
National Vaccine and Serum Institute	Dr. Yu Liu,	Sanjianfang nanli, District Chaoyang, Beijing City, PR China 100024
Liaoning Chengda Biotechnology CO., LTD	Prof. Yingjie Sun	No.1 Xinfang Street, Hunnan New District, Shenyang city, PR China 110179
Guangzhou Promise Biological Products Co., Ltd	Dr. Chunhui He	Wanbao industrial base, Zhong Village, District Panyu, Guangzhou City, PR China 510010
Jilin Maifeng biological pharmaceutical Co., Ltd	Prof. Changlin Zhu	No. 3088, Chang-Ji South district two, Changchun City PR China 130018
Ningbo Rongan biological pharmaceutical Co., Ltd.	Dr. Juan Jin and Dr. Wei Yue	21 ChuangYe Avenue, Western Free Trade Zone, Ningbo City, PR China 315800
Yisheng biopharma Co.,Ltd.	Dr. Wenquan Su	Cailuo industrial zone, District Xinchengzi, Shenyang City, Liaoning, PR China 110131
Hualan Biological Engineering Inc.	Dr. Chuan Jing	Jia No.1, Hualan Ave., Xinxiang City, He'nan, PR China, 453003
Changchun Institute of Biological Products	Prof. Xiuxia Guo and Dr. Li Miao	No. 3456, Xi'an Road, Changchun City, Jilin, PR China 130062
National Institute for Food and Drug Control	Dr. Shouchun Cao	No. 2, Tiantan Xili, District Chongwen, Beijing City, PR China, 100050

diluted DNA bulk solution of Vero cells. During the process, the filling precision was carefully monitored by weighing ampoules filled at the beginning, middle and end stages with a balance (0.0001 g). The ampoules were freeze-dried, nitrogen filled, and heat sealed. The final ampoule contains 20 ng Vero cell DNA reference. The general features, which include appearance, time for reconstitution, pH and residual moisture according to Ch.P, were also tested.

Applicability validation of Vero cell DNA reference. A total of 10 qualified laboratories and domestic vaccine manufacturers were chosen to verify the applicability of Vero cell DNA reference for hybridization assay. The dot blot technique was employed by all participants, and the protocol was based on Ch.P and and instructions from DIG High Prime DNA Labeling and Detection Starter Kit I (Roche).[10] Key parameters for the hybridization included: (1) the probe was prepared with fragments of Vero Cell DNA (200 to 1,000 bp) after ultrasound shearing; (2) the hybridization temperature was set to 45°C; (3) three blind samples of low, medium and high DNA content were also included. To control the reproducibility of the assay, a Standard Operation Procedure SOP was formulated to include a detailed description of the experimental procedure (download freely from: http://www.biosky.org/sop/DNA-blot.pdf, or email the author for detail).

Sensitivity test. An ampoule of Vero cell DNA reference was reconstituted with 0.5 mL distilled deionized water. The DNA was extracted using phenol-chloroform method.[10] Next, the extracted DNA was denatured by boiling for 10 min and placed on ice immediately for 2 min. After a serial dilution, the DNA was carefully blotted onto nylon membranes (10 ng, 1ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg, 1 pg and 0.1 pg per dot), and the TE buffer (0 pg per dot) been as negative control. After hybridization, the signal intensity of each spot was recorded. Further analysis was performed to validate the linearity between DNA quantity and signal intensity.

Stability test. Ampoules of Vero cell DNA reference were placed at 37°C for 2 or 4 weeks (accelerated destruction test). These samples were subsequently used in parallel with ampoules stored at -20°C for dot blot hybridization. The differences in signal intensity were compared between samples treated with different temperatures.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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